REMARKS

Claims 1–28 and 30–51 are pending in this application. Claims 3–5, 27, 28, 30, 33–35, 41, 42, 44, and 47 are allowed. By this Amendment, claims 31 and 32 are amended, and claim 51 is added. Support for the amendments to the claims may be found, for example, in the original claims and specification. No new matter is added.

In view of the foregoing amendments and following remarks, reconsideration and allowance are respectfully requested.

I. Allowable Subject Matter

The Office Action indicates that claims 10–13 recite allowable subject matter. Specifically, these claims are indicated as allowable if rewritten in independent form to include all of the features of the base claim and any intervening claims. Applicants appreciate this indication of allowability, but respectfully submit that at least claim 1, from which these claims depend, and the other claims depending from claim 1 are allowable for at least the reasons indicated below.

II. Rejection Under 35 U.S.C. §102/§103

The Office Action rejects claims 1, 2, 6-9, 14-18, 25, 36-40, 43, and 48-50 as being anticipated by or, in the alternative, under 35 U.S.C. §103(a) as obvious over Wallace. Applicants respectfully traverse the rejection.

Wallace teaches an allele-specific polymerase chain reaction for the detection of sickle cell β-globin in genomic DNA comprising performing PCR in the presence of one primer able to form a mismatched base-pair with the template and in the presence of dNTP(s) at a total concentration of 0.02 g/L (0.1 mM each), followed by electrophoresis (Wallace, col.1, lines 10-13 and 18-19; col.2, lines 21-25 and 53; and col.4, lines 1-2).

The invention as claimed at least differs from Wallace in that (1) the compound that is able to undergo a specific interaction with a mismatched base pair is used at a combined

concentration of at least 10 g/L, which 500 times the concentration used in Wallace, and (2) this compound is selected from a specific group of compounds.

Contrary to the assertions of the Office Action, a skilled artisan never would have considered it obvious to modify the teachings of Wallace by switching from a concentration of 0.02 g/L to a concentration of 10 g/L. Indeed, as indicated in the enclosed Annexes I and II, one of skill in the art would have understood that too high of a concentration of dNTPs causes an inhibitory effect on the PCR process.

More specifically, in the enclosed excerpt from Molecular Cloning – A Laboratory Manual (Sambrook, 3rd Ed. 2001, Tome 2 (Annex I)), it states that a concentration dNTPs of more than 4 mM (combined concentration of 7.79 g/L) is inhibitory. In Annex II (from www.med.yale.edu/genetics/ward/tavi/p13.html), it indicates that the best result are achieved at a concentration of dNTP ranging from 200 to 400 µM (combined concentration of 0.39 to 0.78 g/L), which is very far below the claimed concentration.

This is also confirmed by the Perkin Elmer Cetus datasheet, in which the mentioned recommended assay conditions require a concentration for each dNTP of 200 µM.

Therefore, it would not have been obvious to one of skill in the art at the time of the invention to increase the concentration of dNTP(s) taught by Wallace from 0.02 g/L to 10 g/L, as this concentration would have been considered deleterious to perform the PCR. Also, Applicants submit that a 500-fold increase in concentration cannot reasonably be considered an "optimization." Certainly, the record does support a finding that Applicants' claimed concentration falls within the ranges taught by the applied references, or that the discovery of the claimed concentration is a result of routine experimentation. In fact, as demonstrated above, conventional wisdom in the art taught away from such a high concentration.

In addition, Wallace relates only to the use of **mononucleotide** dNTP and is totally silent with respect to any oligonucleotide of less than 5 nucleotides, of nucleosides, of bases, or mixture thereof as claimed in several of the claims.

For at least these reasons, Wallace does not anticipate, and would not have rendered obvious the rejected claims. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

III. Rejections Under 35 U.S.C. §103

The Office Action rejects claims 19–24, 26, 31, 32, 45, and 46 over Wallace in various combinations with other references. The Office Action provides no suggestion or basis for concluding that any of the other references overcome the above-noted deficiencies of Wallace, such as the specific concentration of compounds to be used within the claimed invention as well as the specific nature of the compounds. Accordingly, claims 19–24, 26, 31, 32, 45, and 46 are patentably distinct from the applied combinations of references at least because of the above-discussed deficiencies. Reconsideration and withdrawal of the rejections are respectfully requested.

IV. New Claim

By this Amendment, claim 51 is added that corresponds to claim 9 rewritten in independent form. Examination and allowance of claim 51 are respectfully requested.

V. Conclusion

In view of the foregoing, it is respectfully submitted that this application is in condition for allowance. Favorable reconsideration and prompt allowance of the application are earnestly solicited.

Should the Examiner believe that anything further would be desirable to place this application in even better condition for allowance, the Examiner is invited to contact the undersigned at the telephone number set forth below.

Respectfully submitted,

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Registration No. 57,771

WPB:JRB

Attachments:
Annex I
Annex II

Date: November 16, 2010

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ANNEX I 1/1

Except from : MOLECULAR CLONING - A LABORATORY MANUAL
3rd Ed. 2001, Sambr. & RUSSEL
SAMBROOK & RUSSEL

success or failure of PCR protocols, it is ironic that the guidelines for their design are largely qualitative and are based more on common sense than on well-understood thermodynamic or structural principles. Compliance with these empirical rules does not guarantee success. Disregarding them, however, is likely to lead to failure. For more information, please see Design of Oligonucleotide Primers for Basic PCR on p. 8.13.

In certain situations, it may be desirable to amplify several segments of target DNA simultaneously. In these cases, an amplification reaction termed "multiplex PCR" is used that includes more than one pair of primers in the reaction mix. For further details on this variation, please see the information panel on MULTIPLEX PCR at the end of this chapter. Standard reactions contain nonlimiting amounts of primers, typically 0.1–0.5 μM of each primer (6×10^{12} to 3×10^{13} molecules). This quantity is enough for at least 30 cycles of amplification of a 1-kb segment of DNA. Higher concentrations of primers favor mispriming, which may lead to nonspecific amplification.

Oligonucleotide primers synthesized on an automated DNA synthesizer can generally be used in standard PCRs without further purification. However, amplification of single-copy sequences from mammalian genomic templates is often more efficient if the oligonucleotide primers are purified by chromatography on commercially available resins (e.g., NENSORB, NEN Life Science Products) or by denaturing polyacrylamide gel electrophoresis, as described in Chapter 10, Protocol 1.

- ***Deoxynucleoside triphosphates (dNTPs).*** Standard PCRs contain equimolar amounts of dATP, dTTP, dCTP, and dGTP. Concentrations of 200–250 μM of each dNTP are recommended for *Taq* polymerase in reactions containing 1.5 mM $MgCl_2$. In a 50-μl reaction, these amounts should allow synthesis of ~6–6.5 μg of DNA, which should be sufficient even for multiplex reactions in which eight or more primer pairs are used at the same time. High concentrations of dNTPs (>4 mM) are inhibitory, perhaps because of sequestering of Mg^{2+}. However, a satisfactory amount of amplified product can be produced with dNTP concentrations as low as 20 μM — 0.5–1.0 pmole of an amplified fragment ~1 kb in length.

  Several manufacturers (e.g., Boehringer Mannheim) sell stocks of dNTPs that are made specifically for PCR. These stocks are free of pyrophosphates that may inhibit PCR and are adjusted with NaOH to a pH of ~8.1, which protects the dNTPs to some extent from damage during freezing and thawing. To avoid problems, stocks of dNTPs (100–200 mM) — whether homemade or purchased — should be stored at −20°C in small aliquots that should be discarded after the second cycle of freezing/thawing. During long-term storage at −20°C, small amounts of water evaporate and then freeze on the walls of the vial. To minimize changes in concentration, vials containing dNTP solutions should be centrifuged, after thawing, for a few seconds in a microfuge.

- ***Divalent cations.*** All thermostable DNA polymerases require free divalent cations — usually Mg^{2+} — for activity. Some polymerases will also work, albeit less efficiently with buffers containing Mn^{2+} (please see Thermostable DNA Polymerases below). Calcium ions are quite ineffective (Chien et al. 1976). Because dNTPs and oligonucleotides bind Mg^{2+}, the molar concentration of the cation must exceed the molar concentration of phosphate groups contributed by dNTPs plus primers. It is therefore impossible to recommend a concentration of Mg^{2+} that is optimal in all circumstances. Although a concentration of 1.5 mM Mg^{2+} is routinely used, increasing the concentration of Mg^{2+} to 4.5 mM or 6 mM has been reported to decrease nonspecific priming in some cases (e.g., please see Krawetz et al. 1989; Riedel et al. 1992) and to increase it in others (e.g., please see Harris and Jones 1997). The optimal concentration of Mg^{2+} must therefore be determined empirically for each combination of primers

ANNEX II 1/2

Page designed and maintained by Octavian Henegariu (Email: Tavi's Yale email or Tavi's Yahoo email).

For a complete description of primers, PCR programs and a discussion of the PCR conditions please consult: *Andrologia* 26: 97-106 (1994) and *Biotechniques* 23: 504-511 (1997). Click here to get the Biotechniques paper in PDF format.

Nucleotides (dNTP)

dNTP "instability"

One important observation, coming from experiments with multiplex PCR, is that dNTP stocks are very sensitive to cycles of thawing/freezing. After 3-5 such cycles, multiplex PCR reactions usually did not work well. To avoid such problems, **small aliquots** (2-5 µl) of dNTP (25 mM each), lasting for only a couple of reactions, can be made and kept frozen at -20° C. However, during long-term freezing, small amounts of water evaporate on the walls of the vial changing the concentration of the dNTP solution. Before using, it is essential to **centrifuge** these vials at high speed in a microfuge.

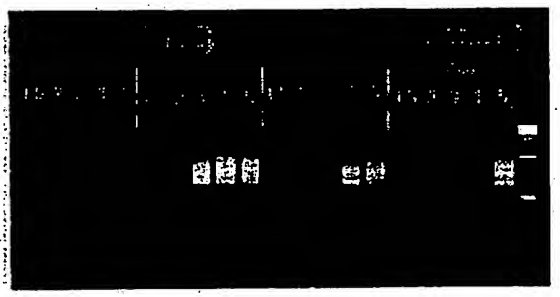
This low stability of the dNTP is not so obvious when single loci are amplified.

Another important observation is that, anytime nucleotides are diluted in water, the solution should be buffered (for example with **10mM Tris pH 7.7-8.0**, final concentration).Otherwise, an acid pH will promote hydrolysis of dNTP into dNDP and dNMP and will render them useless for enzymatic DNA polymerizing reactions.

Relationship between $MgCl_2$ and dNTP concentration (also on page 14)

dNTP concentrations of about 200µM each are usually recommended for the Taq polymerase, at 1.5mM $MgCl_2$ (Perkin Elmer Cetus). In a 25 µl reaction volume, theoretically these nucleotides should allow synthesis of about 6-6.5 µg of DNA. This amount should be sufficient for multiplex reactions in which 5 to 8 or more primer pairs are used at the same time. To work properly (besides the magnesium bound by the dNTP and the DNA), Taq polymerase requires free magnesium. This is probably the reason why small increases in the dNTP concentrations can rapidly inhibit the PCR reaction (Mg gets "trapped")whereas increases in magnesium concentration often have positive effects.

The relationship between the concentration of magnesium and that of the dNTPs was investigated by performing PCR with a degenerate primer in reactions that contained 200, 400, 600 and 800 µM each dNTP, combined with 1.5, 2, 3, 4 or 5 mM $MgCl_2$ (Fig. 34). This test confirmed that any increase in dNTP concentration requires an increase in the concentration of magnesium ions in order for the reaction to work. At 200 µM each dNTP, reaction worked at all magnesium concentrations, but for this primer it worked better at 3 mM (which is about double the recommended magnesium concentration for the amount of dNTP). At 800 µM each dNTP, reaction worked only aboove 3 mM magnesium.



(also shown on page 14)

2/2

Fig. 34. PCR with a degenerate primer at different Mg and dNTP concentrations. Each of the Mg concentrations (1.5, 2, 3, 4, 5 mM) were combined with each of the following dNTP concentrations (each): 200µM, 400µM, 600µM and 800µM. Results indicate that increasing dNTP concentrations require increasing Mg concentrations for the PCR reactions to work.

Common dNTP use in PCR and multiplex PCR

In another test aimed at examining the proper dNTP concentration, a multiplex PCR using primer mixture D was performed. The $MgCl_2$ concentration was kept constant (3mM) while the dNTP concentration was increased stepwise from 50 to 100, 200, 400, 600 and 1200 µM each deoxynucleotide (Fig. 35). The best results were achieved at 200 and 400 µm dNTP; reaction was rapidly inhibited after these values. Lower than usual dNTP concentrations still allowed PCR amplification, but with somewhat less efficiency (lane "50").

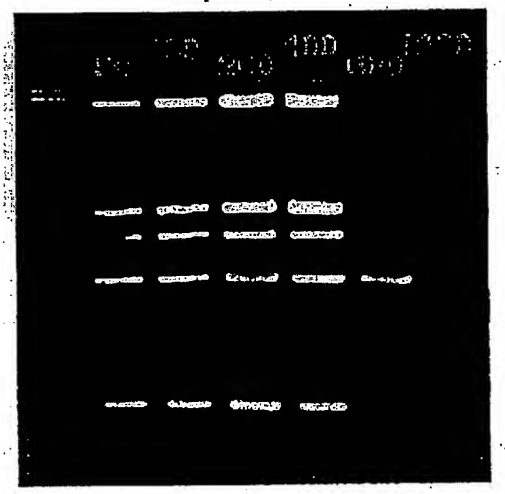


Fig. 35. Multiplex PCR amplification of mixture D in 2x PCR buffer (3 mM Mg) using increasing concentrations of dNTP (50mM, 100mM, 200mM, 400mM, 600mM and 1200mM each). Most efficient amplification is seen at concentrations of 200-400µM each dNTP. Further increase in the dNTP concentration inhibits the reaction when $MgCl_2$ is kept constant.